Metal Affinity Engineering of Proinsulin Carrying Genetically Attached (His)$_{10}$-X-Met Affinity Tail and Removal of the Tag by Cyanogen Bromide

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Received April 15, 1994

An E. coli expression clone coding for human proinsulin, which was fused to NH$_2$-terminal β-galactosidase, was engineered for the separation from host proteins by introducing peptide devices, and for the sequential removal of the fused polypeptide by cyanogen bromide in front of the NH$_2$-terminal residue (methionine) of the human proinsulin gene. Short synthetic genes encoding oligopeptide residues including (Glu)$_n$ (His)$_n$ (Trp)$_n$ and (Ser)$_n$ (n = 10 or 11), which have certain characteristic physical properties such as metal-affinity, polarity, hydrophobicity, and hydrophilicity, respectively, were inserted at the junction region of the gene fusion. Interestingly, it was found that among the oligopeptides, the oligohistidine residue as an affinity-tag has greatly facilitated the procedures for FPI purification, particularly in the manner of selective metal-affinity precipitation. The chelating peptide covering the NH$_2$-terminal beta-galactosidase portion could then be removed simply after purification to generate a protein with the natural amino acid sequence of proinsulin by cyanogen bromide.

Recent progress in recombinant DNA techniques has resulted in the industrial scale of productions of rare and valuable proteins from microorganisms and higher organisms. But in the conventional sense, their purification procedures are still too laborious and inefficient to obtain high purity of the final recombinant proteins before their use. 1-10 Hence, major efforts are still being done for improving the separation efficiency and simplifying the downstream processes. In fact, if simple and efficient purification methods like pH precipitation, organic solvent extraction, metal-affinity precipitation, or (and) crystallization are applicable to purification processes of bioactive proteins, they might be the most desirable choice for separations because they can be easily extended to industrial practices, but such methods, specific to certain biomolecules, are generally not much useful for biomolecule separations.11-13

With respect to those recombinant proteins, the use of affinity-linked purification methods have been examined in the large-scale production of bioactive peptides and proteins by Porath et al.,14 taking advantage of the interaction between biomolecules and transition metals. In affinity-linked purification, metal ions are immobilized on a hydrophilic support derivatized with iminodiacetic acid groups, which chelate metal ions. This affinity resin, when charged with nickel ions (Ni$^{2+}$-NTA adsorbent), has a remarkable selectivity for proteins and chelating peptides (CP) containing neighbouring histidine residues. Metal-chelating ligands such as iminodiacetic acid charged with Ni$^{2+}$12 or Zn$^{2+}$16,17 and nitritrolactric acid charged with Ni$^{2+}$18 have been used, together with various histidine-containing affinity tails.

Removal of the chelating peptide to generate the protein of interest is central to the utility of metal affinity purification. Cleavage reactions have been done out on many recombinant fusion proteins to liberate the desired protein19-21 and can be applied to CP-proteins as well. The cleavage reaction of choice will depend on the composition and sequence of the protein of interest. For instance, if the protein lacks tryptophan residues, then a tryptophan oxidative cleavage reaction22 could be used to remove the Trp-containing peptide to generate the desired protein after purification using the metal affinity method. Tryptophan oxidative cleavage has been used to cleave insulin-like growth factor I from the leader sequence of a fusion protein expressed in E. coli.20 Cleavage with hydroxylamine at an asparagine-glycine peptide bond has also been used to liberate insulin-like growth factor I from an expressed fusion protein.22,23 Cyanogen bromide is another reliable cleavage reagent used in recombinant fusion proteins devoid of methionine residues in the protein sequence of interest. Enzymatic cleavage reactions have also been developed for the production of recombinant proteins, such as human growth hormone,21 which could be incorporated into the design of CP-protein by including an intervening enzyme substrate cleavage site between the CP and the desired protein.

On the other hand, proinsulin was particularly appropriate for separation using CP because it contains a histidine B10 zinc binding site capable of binding transition metals, such as nickel.24 The crystal structure of insulin shows the imidazole group of histidine B10 is exposed to the solvent.24 This group is presumably responsible for proinsulin binding to immobilized Ni(II) in those experiments. Recently, Smith

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Abbreviations: FXPI, fused-(oligo X)-proinsulin; FHPI, fused-(oligohistidine)-proinsulin; FSPI, fused-(oligoasparagine)-proinsulin; FPEI, fused-(oligoglutamate)-proinsulin; FWPI, fused-(oligo tryptophane)-proinsulin; FPI, fused-proinsulin; SPI, sulfonate-proinsulin; NFX, NH$_2$ terminus-fused-peptide; NFH, NH$_2$ terminus-fused-oligohistidine-peptide; BSA, bovine serum albumin; PMSE, IPTG, isopropyl-β-D-thiogalactopyranoside.

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et al. demonstrated that a simple metal binding site could be engineered into a polypeptide and exploited to make the purification of that polypeptide easier. The addition of a chelating peptide, Met-His-Trp, on the NH₂ terminus of proinsulin led to stronger binding to immobilized Ni(II) and allowed it to be separated from proinsulin. Since the rest of the proinsulin sequence does not contain any methionine residues, the desired proinsulin derivative could be separated from the trpLE polypeptide fragment by cyanogen bromide cleavage. However, they still suffered from deletion of the extra dipeptide His-Trp in the desired proinsulin derivative and this problem should be solved.

Proinsulin was synthesized by recombinant DNA methods using Escherichia coli fermentations as reported by Goeddel et al. from the Genentech Company and the City of Hope National Medical Center, and also from Eli Lilly and Company, it was generally accepted that proinsulin could be rapidly and completely cleaved by a combination of pancreatic trypsin and carboxypeptidase B in vitro to yield intact insulin, the C-peptide, and free arginine and lysine. Thus, human insulin derived from recombinant technology which was designated biosynthetic human insulin, was found to be chemically, physically, and immunologically equivalent to pancreatic human insulin and biologically equivalent to pancreatic human insulin. The general strategy for preparing human insulin by genetic approaches has been reviewed by several investigators.

For a previous paper, we synthesized the proinsulin gene chemically and it was stably expressed in E. coli, forming typical inclusion bodies. In this report, we describe the construction of expression clones coding for human proinsulin fused to NH₂-terminal beta galactosidase and oligopeptides such as oligo-histidine, oligo-tryptophane, and oligo-serine as well as oligoglutamate, and their high-level expression in E. coli. The metal-affinity separation of proinsulin was also described in relation to peptide engineering of the recombinant fused proteins. Particularly, among oligopeptide residues, the oligohistidine residue in FPI had a high affinity for transition metal ions, such as, copper ion, manganese ion, nickel ion, and zinc ion, through which could develop an practical method in proinsulin separation, using the simple metal-affinity precipitation under controlled conditions. Finally, the sequential removal of the fused extra polypeptide, a key process of proinsulin production, could be done by cyanogen bromide cleavage in front of the NH₂-terminal residue (methionine) of the human proinsulin gene.

Materials and Methods

Construction of plasmid pPRO(X) and its expression. The proinsulin gene had been previously constructed in our laboratory (Lim et al., 1987). The oligonucleotides corresponding to oligopeptide gene (Fig. 1) were synthesized chemically by an automatic DNA synthesizer (Applied Biosystem Co. model 381A). The phosphorylated synthetic genes were cloned into the BglII sites of the plasmid pPRO to give the plasmid pPRO(X). The plasmids with FXPI genes were used to transform E. coli MV1184. Transformants were analyzed by restriction mapping and DNA sequencing. Cells harboring pPRO(X) were initially grown at 37°C in LB medium with 100 µg/ml ampicillin, and then cells were put into minimal medium. When the cell population reached O.D. 0.7 (λ = 600 nm), the FXPI gene was induced in the presence of IPTG (1 mM) for 2 hours more before harvest.

Purification of FXPI. The cell mass (25 g/liter medium) grown in minimal medium was recovered by centrifugation (4,000 x g, 10 min, 4°C). It was lysed by a French press (12,000 psi) in 50 mM Tris·HCl buffer (pH 8.0) containing 100 mM NaCl, 10% (w/v) sucrose, and 0.2 mM PMSF. After the inclusion bodies and cell debris were centrifuged down (15,000 x g, 15 min, 4°C), the precipitate was washed with the above buffer solution containing 0.5% (v/v) Triton X-100 to remove minor cellular proteins. The inclusion bodies in the precipitate were fully dissolved by 7 M urea buffer solution.

CNBr Cleavage of FXPI. Crude FXPI (5 mg/ml) in 70% (v/v) formic acid was kept at room temperature under a nitrogen atmosphere. CNBr (100 x molar ratio to protein) was added. The reaction was kept in the dark for 48 hours and stopped by water dialysis.

Oxidative sulfation. After CNBr cleavage of FPI, the dialysate (5 mg/ml) of the product mixture was sulfonated by sodium tetraiodotride (200 mg/g of substrate) and sodium sulfite (200 mg/g of substrate) in aqueous solution (pH 9.0, titrated with NH₄OH) at room temperature under a nitrogen atmosphere for 6 hours. The reaction was quenched by adding excess water. The reaction solution was directly used for metal-affinity precipitation.

Purification of SPI. The reaction mixture was treated with manganese ion (20 x molar ratio). Most of the proteins containing a metal-affinity tag were precipitated in the solution except SPI. After centrifugation (15,000 x g, 15 min, 4°C), the supernatant was dialyzed several times to remove salts, and freeze-dried. To check for the presence of metal salts in the SPI, dithizone in chloroform was used to treat the solution of digested SPI at pH 11. Any absorbance at 650 nm indicated the presence of manganese ion in the solution.

Analytical methods. The purity of proteins was analyzed by SDS-polyacrylamide (14%) gel electrophoresis by the method of Laemmli. Proteins were measured by the Lowry method using bovine serum albumin (BSA) as a standard. The amino acids of the N-terminus of SPI were sequenced by an Applied Biosystem 470A peptide sequencer. Amino acid composition was analyzed through hydrolysis of SPI with vaporized 6 N HCl in a closed system, and followed by standard analysis of amino acid composition with Beckman HPLC System Gold, which was also used to certify the purity of proteins. Western-blot analysis was used to confirm the presence of proinsulin fragment after the CNBr reaction and after metal-affinity separation of SPI. A Sebia Co. densitometer was used for the measurement of proteins on SDS-polyacrylamide gels.

Results

Cloning and expression of fused-(oligopeptide)-proinsulin (FXPI) genes

Short synthetic DNA cassettes (Fig. 1) encoding oligopeptides, (His)ₙ, (Trp)ₙ, (Ser)ₙ, and (Glu)ₙ (n = 10 or 11) were designed, in which both protruding ends had BglII cohesive ends. The synthetic genes were inserted into the BglII site of the fused-proinsulin gene in the plasmid pPRO, which was constructed as an expression vector containing the tac promoter, the fused human proinsulin genes, and the transcription terminator rrrB gene. Plasmids pPRO(X) (X = H, W, S, E) with the oligopeptide genes were used to transform E. coli MV1184 and were analyzed by mapping and DNA sequencing (Fig. 2). Each clone was grown in LB medium with ampicillin (Amp). After the induction of gene expression with isopropyl-β-D-thiogalactopyranoside (IPTG), the cellular proteins of the cloned cells were analyzed by SDS–PAGE and a densitometer. As exemplified in Fig. 3a, each clone containing the oligopeptide genes, pPRO(X), directed the synthesis of FXPI. Particularly, the clones for fused-(oligohistidine) proinsulin (FHIPI), fused-(oligoserine) proinsulin (FSPI), and fused-(oligoglutamate) proinsulin (FEPI) provided high level of the expression, more than 43% of cellular proteins (Fig. 3b). However,
Fig. 1. Plasmid Construction for the Peptide-Engineering of Proinsulin Separation by Introducing the Oligopeptide Genes.

The plasmid, pPRO(H), for example, is made of the regulatable tac promoter, proinsulin gene fused with the N-terminal portion of modified β-galactosidase gene (MB), transcription terminator rmb gene (TIT2), and β-lactamase gene (Amp).

Fused-(oligotryptophan) proinsulin (FWPI) provide low level of the expression with unknown reasons (data not shown).

Purifications of FXPI

FXPI except PWF1 was obtained from the inclusion bodies in the cell mass. At first, E. coli cells were lysed by a French press in the solution containing 100 mM NaCl and 50 mM Tris–HCl buffer, pH 8.0. The precipitation was then collected by centrifugation, and followed by the precipitation dissolution with 7 M urea buffer. Finally, dialysis gave crude FXPI.

Solubility of FXPI

Each FXPI was dissolved in aqueous solution at different pHs. Recombinant proteins FEPI, FSPI, FHIP, and FPI were precipitated at pH 4.5, and the 3 proteins other than FEPI were precipitated even at pH 6.0. However, FEPI was dissolod at pH 6.0, indicating that FEPI had 10 additional anionic residues in itself, which changed the pI 4.65 to 4.25 (data not shown). These suggested that these pH changes of FXPI might be applicable to a large scale of separation of recombinant proteins.
Fig. 3. a) Analysis of Total Cell Fraction of E. coli Transformants by 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE). M, standard molecular mass markers of ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (18 kDa). C, E. coli host cell. P, E. coli harboring plasmid pRRO induced with IPTG. H, E. coli harboring plasmid pRRO(H) induced with IPTG. 26 K and 28 K proteins indicate the β-galactosidase-fused proinsulin and β-galactosidase-(His)₆-fused proinsulin, respectively.

b) Denistogram of SDS PAGE for FPI Gene Expression.

A major peak indicated FHPI was >43% of total cellular proteins. Arrow indicates the position of the fused protein (28 K) and arrowhead indicates percentage of the fused protein in total proteins.

Table: Molar Ratio of Metal Ions Needed for FHPI Precipitation

<table>
<thead>
<tr>
<th>M²⁺ ion/FHPI</th>
<th>Fe²⁺</th>
<th>Zn²⁺</th>
<th>Mg²⁺</th>
<th>Ni²⁺</th>
<th>Mn²⁺</th>
<th>Cu²⁺</th>
<th>Co²⁺</th>
<th>Ca²⁺</th>
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<tr>
<td>Molar ratio</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;200</td>
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molar ratios of metal ions for their precipitations were different (Table). For example, a 20 molar ratio of manganese ion was needed for FHPI precipitation, which indicated that one histidine residue bound approximately two molecules of metal ions. Further characterization and biochemical properties of FEPI, FWPI, and FSPI will be published elsewhere.

Immobilized metal-affinity chromatography (IMAC) of FHP

Nickel ion was immobilized on Sepharose resin to examine selective separation of FHPI,13-15,18 and when crude FHPI was applied to IMAC it was eluted with an imidazole gradient. Both experiments showed that FHPI with the affinity tag of metal ion was eluted very slowly (Fig. 5), compared to that of cellular proteins. It could be that oligohistidine has high binding ability to nickel ion on the resin. Therefore, a metal-affinity tag might be highly suitable for the chromatographical separation of certain recombinant proteins.

Cyanogen bromide (CNBr) cleavage of FXPI and oxidative sulfonation

FXPI was treated with cyanogen bromide in formic acid to release the intact proinsulin molecules from FXPI.13}
Fig. 5. Chromatogram of FHPI Purification by Imidazole Metal Affinity Chromatography (IMAC).

Inclusion bodies of FHPI in precipitate were fully dissolved by adding 7 M Urea buffer solution (final conc.) as described in Materials and Methods and the dissolved FHPI was diluted 10 times with 50 mM Tris-HCl (pH 8.0). FHPI was applied to a Tris-HCl buffer on nickel-binding column in FPLC system with imidazole gradient at pH 8.0. FHPI were selectively isolated in range of 0.18 to 0.25 M imidazole on IMAC. ■, protein; □, imidazole concentration (M).

Fig. 6. Gradient SDS-PAGE (12 to 16% gel) Analysis of Metal Affinity Precipitation.

Lane C was crude extract of FHPI from the inclusion bodies. Lane B was the product mixture of CNBr cleavage in 70% formic acid. Lane S and P were supernatant and precipitate, respectively, which were obtained from 5 Molar equivalent NiCl₂ treatment at pH 8 to SPI mixture of S-sulfonation, after CNBr cleavage of FHPI. Lane M was standard molecular weight markers including carbonic anhydrase (29 K), hen egg white lysozyme (14 K), bovine trypsin inhibitor (6 K), and insulin α,β-chains (3 and 2 K). Large arrowhead indicates the position of the fused protein (28 K).

Because one methionine residue inside FXPI is just before the proinsulin portion, the cleavage reaction yielded two peptide fragments, the proinsulin molecule and an N-terminus-fused peptide (NFX) including the affinity tag (Fig. 6). After CNBr cleavage, the cleaved products comprising FHPI, N-terminus-fused-oligohistidine peptide (NFH), and proinsulin were sulfonated in the presence of tetrathionate, sodium sulfite in aqueous solution. Then, chemical reagents were simply removed by repetitive dialysis.

Metal-affinity precipitation of S-sulfonated proinsulin (SPI)

Sulfonated product mixture was treated with nickel or manganese ions to precipitate sulfonated FHPI (SFHPI) and NFH, in which metal-affinity tags are attached. Interestingly, most of the protein mixture was cleanly precipitated except negatively charged SPI and was centrifuged out. The supernatant was repetitively dialyzed to remove the salts, and the purity of SPI was examined in SDS-PAGE. As shown in Fig. 6, this simple metal-affinity precipitation gave more than 95% SPI purity. It greatly simplified the process of insulin production by skipping two consecutive separation steps, i.e., separations of inclusion body and proinsulin after the cleavage reaction. Definitely this method can be readily applied to industrial scale proinsulin separation.

Discussion

In insulin production, a number of steps are followed to purify its intermediate products, which are time-consuming and costly. A solution for such biomolecule separations might be the physical property engineering of the recombinant proteins through recombinant DNA techniques. Simple separation devices was made by inserting certain designed gene fragments into FPI. Lately, there were some efforts by the peptide engineering of recombinant proteins to facilitate their separations. In this report, the FPI gene was redesigned to introduce the characteristic physical properties that might be highly applicable to the process of proinsulin separation. Synthetic genes for the oligopeptides were designed, made, and inserted into the FPI gene in plasmid pPROI(X). Interestingly, the insertion of the synthetic genes into FPI gene had positive effects in their gene expressions for unknown reasons. We examined the physical properties of their gene products: the solubility in aqueous and organic phases, the pH precipitation, and the metal affinity. FPI having the oligopeptide residues had noticeable changes in their physical properties. For example, the negatively charged FEPI in acidic pH conditions has a substantial change in its pI value (data not shown). It appears to be a critical device for introducing new and efficient separation methods, using the high solubility in aqueous and polar media.

One of the outstanding findings is that this peptide engineering could make a selective separation device inside FPI. Of them, FPI and FEPI were not sensitive to metal affinity precipitation (data not shown). However, FHPI was easily purified by metal-affinity chromatography and metal-affinity precipitation. Such a selective affinity by metal ions could be attributed to the involvement of a transient coordination complex, as described by many authors. When metal ions were added to FHPI in aqueous solution, the molar ratio of metal ions over FHPI for the precipitation turned out to be dependent upon the metal ion used. The molar ratio of heavy metals is less than alkali metal ions, which is attributed more or less to the ability of complex formation and the limitation of their accessibility to the oligohistidine residue inside FHPI. Particularly, manganese ion appears to be intermediate in the mole ratio needed for its precipitation.

Among metal ions tried for FHPI separation, nickel and manganese ions have better selective affinity than those of other metal ions, which might be explained by the binding ability of the surface of the His-X3-His site to the chelated metal ion. It provides the basis for analysis of metal-binding properties by partitioning experiments with metal-chelating.
polymers. Overall the separation method for FHPI by metal affinity has an outstanding performance.

It was recently reported that immobilized metal affinity chromatography (IMAC) for purifying the insulin fused-protein by introducing a tripeptide, Met-His-Trp was used with considerable improvement of binding selectivity by Smith et al. Since the proinsulin sequence does not contain any methionine residues, the desired proinsulin derivative could be separated from the trpLE' polypeptide fragment by cyanogen bromide cleavage. However, they still suffered from depletion of the extra dipeptide His-Trp attached to the NH$_2$-terminal phenylalanine residue in the desired proinsulin derivative, even though they used a chemical cleavage method to remove the dipeptide.

When crude FHPI was analyzed by nickel-immobilized chromatography, FHPI showed strong affinity for nickel ion with high selectivity (Fig. 5). It could be thought that the metal-affinity to proinsulin might be dependent upon certain conditions for the formation of transient coordination complex. In this study, further chromatographical approaches for proinsulin separation were ruled out due to its low adaptability to industrial practices. Cyanogen bromide cleavage of FHPI gives proinsulin and the fused portion of FHPI, and was subsequently sulfonated before refolding the proinsulin. The metal-affinity tag is only located inside the fused portion of protein, not inside the proinsulin, which is now negatively charged by six cysteine residues. Under this circumstance, the metal-affinity method could reach the maximum efficiency for proinsulin purification because of the high solubility of sulfonated proinsulin molecule in aqueous solution. When manganese ion was treated with a solution of the reaction mixture, the result was extraordinarily clean and highly selective (Fig. 6). So proinsulin molecules remained in the supernatant and the rest of proteins were clearly precipitated out. It indicates that the strength of the solubilization of sulfonated proinsulin molecules surpassed the metal-binding strength to proinsulin. Overall, this metal precipitation step is exceptionally simple and good enough to have highly purified SPI before its folding step, which might be the most desirable separation technique in insulin production.

In this study, peptide engineering of FPI by introducing oligopeptides as a metal-affinity tag showed to be successfully managaged to purify SPI. The change of physical properties of recombinant proteins might be very useful and powerful for industrial purification of macromolecules. Finally, the sequential removal of the fused extra polypeptide, a key process of proinsulin production, could be successfully done by cyanogen bromide cleavage in front of the NH$_2$ terminal residue (methionine) of the human proinsulin gene.

Acknowledgment. This study was in part supported by grants from Ministry of Science and Technology (MOST), Korea.

References